Alterations of nuclear envelope and chromatin organization in mandibuloacral dysplasia, a rare form of laminopathy

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MATERIALS AND METHODS

Patient samples. Human fibroblasts were isolated from skin biopsies (dorsal forearm) obtained from three MADA patients and from three control subjects. All biopsies were obtained under institutionally approved protocols (Tor Vergata University, Rome; Gaetano Rummo Hospital, Benevento; Italian Dermatological Institute, Rome, Italy). MADA patients (MADA-1, female; MADA-2 and MADA-3, males) underwent a skin biopsy at 18 (MADA-1), 35 (MADA-2), and 50 yr of age (MADA-3). All MADA patients were homozygous for the R527H mutation, and they showed the same clinical phenotype.
without important differences. The three control biopsies were age and sex matched. Fibroblast cultures were established by mechanical and enzymatic methods and cultured in Dulbecco’s modified Eagle’s-F12 medium (Cambrex) supplemented with 15% fetal bovine serum (Cambrex) and antibiotics. The passage number of each cell type was recorded, and cells were analyzed when passages 2 and 6.

**Immunofluorescence staining.** Human fibroblasts were grown on coverslips coated with poly-l-lysine, rinsed in PBS, and fixed for 10 min with 4% (wt/vol) paraformaldehyde (in PBS). Cells were permeabilized for 5 min with 0.1% Triton X-100 in 100 mM Tris·HCl, pH 7.5. Incubation with affinity-purified rat anti-HIP1β IgG (Mac 353) (46) and rabbit anti-Me9H3 IgG (13) was carried out at room temperature for 1 h. For LBR, prelamin A, and emerin detection, cells were fixed and permeabilized with cold methanol at −20°C for 7 min, rinsed in PBS, and incubated overnight at 4°C with polyclonal rabbit anti-LBR IgG, polyclonal goat anti-prelamin A antibody (Santa Cruz, sc-6214), or anti-emerin mouse monoclonal antibody (Novocastra). Cy3-conjugated anti-goat IgG (DAKO), FITC-conjugated anti-mouse IgG (Sigma), and Texas Red anti-mouse IgG (Calbiochem) were used as secondary antibodies. Hoechst 33342 dye was used at 300 ng/ml. Samples were examined with a Leica fluorescence microscope equipped with a CCD camera. Acquired images were deconvolved using Leica Qfluoro software and processed using Adobe Photoshop.

**Western blot analysis.** Human fibroblasts were lysed in ice-cold 10 mM Tris·HCl buffer, pH 7.4, containing 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMFS), and 10 μg/mL aprotinin, leupeptin, and pepstatin. For prelamin A detection, 1% SDS was added to the extraction buffer. Blots were probed with anti-lamin A/C (mouse monoclonal; Novocastra), anti-prelamin A (goat polyclonal; Santa Cruz, sc-6214), or anti-emerin mouse monoclonal antibody (Novocastra). Cy3-conjugated AffiniPure donkey anti-rabbit IgG (Jackson), FITC-conjugated anti-goat IgG (DAKO), Cy3-conjugated anti-mouse IgG (Sigma), and Texas Red anti-mouse IgG (Calbiochem) were used as secondary antibodies. Hoechst 33342 dye was used at 300 ng/ml. Samples were examined with a Leica fluorescence microscope equipped with a CCD camera. Acquired images were deconvolved using Leica Qfluoro software and processed using Adobe Photoshop.

**Electron microscopy.** Cell pellets from confluent control and MADA fibroblast cultures were fixed with 2.5% glutaraldehyde-0.1 M phosphate buffer, pH 7.6, for 1 h at room temperature. After treatment with 1% osmium tetroxide in veronal buffer for 1 h, pellets were dehydrated in an ethanol series and embedded in Epon resin. Thin sections stained with uranyl acetate and lead citrate were observed with a Philips EM 400 transmission electron microscope, operated at 100 kV. At least 200 nuclei per sample were observed. Statistical analysis was performed by counting nuclei from three different preparations per each examined sample.

**Preparation of nuclear and cytoplasmic fractions and Western Blot analysis.** Human fibroblasts were washed twice in PBS, scraped, and collected. The nuclear and cytoplasmic fractions were prepared by suspending cells in 0.3 ml of hypotonic isolation buffer [IB; 10 mM Tris·HCl, pH 7.6, 10 mM NaCl, 1 mM MgCl2, protease inhibitor cocktail (1:1,000; Calbiochem), and 0.1 mM PMSF]. Cells were passed through an ice-cold cylinder cell homogenizer, and nuclei were isolated by centrifuging at 4°C for 15 min at 290 g. Nuclear pellets were washed twice with 0.3 ml of IB, incubated for 30 min on ice in modified IB with 1% Triton X-100, and centrifuged at 12,000 g for 15 min to separate the soluble and the insoluble nuclear fractions. The cytoplasmic supernatant, after two subsequent centrifugations for clearing from cell debris, was detergent extracted by adding 0.5% Nonidet P40 (NP40; Sigma) for 30 min on ice and centrifuged twice (12,000 g at 4°C for 15 min). The cytoplasmic pellet was washed twice in NP40-enriched IB buffer by subsequent centrifugations at 12,000 g for 15 min. Equal amounts of soluble (S) and insoluble (I) proteins from nuclei and cytoplasm were separated on SDS-PAGE in 12% acrylamide gels and blotted.

**RESULTS**

**Cellular level of prelamin A is increased in cells of MADA patients.** Fibroblast cultures from a healthy patient aged 35 yr and three MADA patients aged 18 (MADA-1), 35 (MADA-2), and 50 yr (MADA-3) carrying the same R527H LMNA mutation were analyzed. In particular, we examined by Western blot the LMNA products prelamin A, lamin A, and lamin C. We observed accumulation of prelamin A in fibroblasts derived from MADA patients, with a linear increase of protein amount in older patients (Fig. 1, A and B). Lamin A level was unaffected in the younger subject and progressively reduced in fibroblasts derived from the older patients (Fig. 1, A and B). Lamin C level was slightly reduced in MADA-3 fibroblasts.
The expression level of the lamin A/C-binding protein emerin was not altered in MADA fibroblasts (Fig. 1A).

We then analyzed, by double immunofluorescence, the intracellular localization of prelamin A and emerin in control and MADA nuclei. Control cells (Fig. 2A) expressed low amounts of prelamin A that appeared mostly distributed at the nuclear envelope. In marked contrast, MADA nuclei showed accumulation of prelamin A in the nuclear envelope, associated with formation of intranuclear prelamin-labeled structures (Fig. 2, B–D). Emerin was localized at the nuclear envelope of MADA-1 and MADA-2 nuclei (Fig. 2, F and G), while it showed a honeycomb-labeling pattern in MADA-3 cells (Fig. 2H). Partial colocalization of prelamin A with emerin was observed in MADA-1 and MADA-2 nuclei, but colocalization was lost in most MADA-3 nuclei (Fig. 2, J–L).

To further investigate whether the observed accumulation of prelamin A in MADA nuclei is related to changes in the distribution of proteins of the nuclear envelope, we studied the localization of LBR in these cells (Fig. 3). In control fibroblasts, LBR was localized at the nuclear rim (Fig. 3A). A minor percentage of nuclei (7%) showed intranuclear diffuse staining of LBR (Fig. 3B). Interestingly, these cells also presented a higher prelamin A level at the nuclear envelope (Fig. 3, F and G). In MADA cells, on the contrary, LBR distribution was altered in a high percentage of cells. Thus ~50% of MADA-1 nuclei showed both nuclear rim and diffuse nucleoplasmic staining (Fig. 3, C and H). Nucleoplasmic staining, cytoplasmic localization, and a reduced nuclear envelope labeling of LBR was typical of 60% of MADA-2 (Fig. 3, D and I) and 65% of MADA-3 nuclei (Fig. 3, E and O).

**LBR staining of the nuclear envelope is lost in MADA nuclei.**

Fig. 2. Double-immunofluorescence staining of prelamin A and emerin in control and MADA fibroblasts. Prelamin A (A–D) and emerin (E–H) were labeled using specific antibodies. Merged images are shown in I–L.

Fig. 3. Double-immunofluorescence staining of lamin B receptor (LBR) and prelamin A in control and MADA fibroblasts. Control and MADA-1, MADA-2, and MADA-3 fibroblasts were double labeled with anti-LBR (A–E) and anti-prelamin A antibodies (F–J). The DNA was counterstained with Hoechst 33342 (K–O).
65% of MADA-3 cells (Fig. 3, E and J). In some cells, LBR staining at the nuclear rim was almost completely lost (Fig. 3, E and J). Both lamin A precursor accumulation at the nuclear envelope (Fig. 2, B–D) and LBR nucleoplasmic localization (Fig. 3, H–J) increased with patient age. Nucleoporins and lamin B were correctly localized in all examined fibroblasts (data not shown).

**Heterochromatin organization is lost in MADA nuclei.** The ultrastructural morphology of MADA fibroblasts was examined by electron microscopy. This analysis revealed striking nuclear alterations (Fig. 4). In particular, invaginations of the nuclear envelope or thin papillary projections characterized 15–40% of nuclei (Fig. 4, B and C). We observed nuclear dysmorphism, irregular thickness of the nuclear lamina (Fig. 4D), focal absence of peripheral heterochromatin, or complete heterochromatin loss (Fig. 4E). In some nuclei, projections, invaginations of the nuclear envelope, and peripheral heterochromatin loss were simultaneously observed. A minor percentage of nuclei showed scarce density of interchromatin when compared with controls (Fig. 4F). All these alterations were absent in control nuclei (Fig. 4A). The observed nuclear defects were more represented in older MADA cells (Fig. 4G). In particular, complete absence of heterochromatin from the nuclear periphery ranged from 1–2 to 40% (of 200 nuclei examined) according to patient’s age (Fig. 4G).

**Distribution of HP1β and Me9H3 is affected in MADA nuclei.** To test whether nuclear envelope alterations affect the heterochromatin organization, two major structural components of heterochromatin, the HP1β and Me9H3, were studied by double immunofluorescence. In control fibroblasts (Fig. 5, A, E, I, and M), the nuclei were round or ovoid, and the distribution pattern of Me9H3 and HP1β...
appeared dispersed in multiple small foci filling the whole nuclear area. A strong colocalization of both proteins was evident in merged image (Fig. 5M). This pattern appeared partially modified in MADA cells in a way that reflected the age of the patient. In particular, in fibroblasts derived from MADA-1 patient, the nuclear morphology was essentially preserved, although a more distinctive localization of Me9H3 and HP1β/H9252 was detected in 15% of cells (Fig. 5, B, F, J, and N). Interestingly, these cells also displayed a punctuate DNA distribution, visualized by Hoechst staining (Fig. 5B), different from the typically more uniform DNA staining of control cells (Fig. 5A). These fibroblasts accumulated a distinct chromatin structure enriched with heterochromatin proteins, similar to the recently described senescence-associated heterochromatin foci (SAHF) (33). In 5–10% of MADA-2 (Fig. 5, C, G, K, and O) and in 30–40% of MADA-3 nuclei (Fig. 5, D, H, L, and P), a pronounced alteration of the nuclear morphology and a different distribution pattern of the investigated heterochromatin proteins were observed. MADA-3 nuclei showed severe signs of degeneration with multiple invaginations and lobulations. In most of the lobules, the colocalization of the proteins was irremediably lost (Fig. 5P). While Me9H3 was detected inside the papillary extroflessions, no apparent HP1β was present in these area.

To further analyze the biochemical features of the two heterochromatin markers, we fractionated fibroblasts derived from control and MADA patients. We first separated the nuclear and the cytoplasmic fractions in the absence of detergent. Subsequently, the two fractions were Triton extracted and centrifuged to isolate the soluble and insoluble pools from each fraction. After separation, we examined, by Western blot, the distribution of the HP1β/H9252 and Me9H3 in these fractions. In control human fibroblasts, both HP1β/H9252 and Me9H3 are mostly found in the insoluble pool of the nuclear pellet (Fig. 6A, lanes 1 and 2). On the contrary, in fibroblasts derived from MADA-1 and MADA-3 patients, these proteins are differently partitioned and found also in the nuclear soluble pool (Fig. 6A, lanes 3 and 5). The densitometric analysis of HP1β and Me9H3 bands is shown in Fig. 6B. Interestingly, both HP1β and Me9H3 accumulation in the soluble pool of the nucleus increased with patient age, varying from 40–42% in MADA-1 to 48–50% in MADA-3 fibroblasts. It is worth noting that no immunoreactive pro-

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Fig. 5. Double-immunofluorescence staining of trimethylated histone H3 at lysine 9 (Me9H3) and heterochromatin protein-1β (HP1β) in control and MADA fibroblasts. Localization of Me9H3 (green) and HP1β (red) was determined by indirect immunofluorescence on control (C) and MADA-1, MADA-2, and MADA-3 fibroblasts. The DNA was counterstained with Hoechst 33342 (H342; blue). Merged images are shown. Scale bar = 5 μm.
teins were present in the cytoplasm, either in control fibroblasts or in MADA cells (data not shown). The fact that the expression level of HP1β and the methylation state of H3 histone at lysine 9 were not altered, while the molecules were partially solubilized by Triton X-100 treatment of MADA fibroblasts, is consistent with the finding that heterochromatin in these cells is partly unstructured.

To investigate whether destabilization of HP1β and Me9H3 correlates with alteration of the lamina, we analyzed the distribution of prelamin A, lamin A/C, and emerin between supernatant and pellet fractions. In control human fibroblasts, lamin A/C and prelamin A distribute in the nuclear pellet, and emerin is also partly found in the nuclear soluble pool (Fig. 6C, lanes 1 and 2). These three molecules are not differently partitioned in MADA cells (Fig. 6C, lanes 3 and 4).

**DISCUSSION**

In this report, we have documented the presence of nuclear envelope and chromatin alterations in primary cultured fibroblasts from patients carrying a missense mutation in the LMNA gene (R527H) resulting in MADA phenotype. We demonstrated accumulation of prelamin A, altered stability of heterochromatin proteins HP1β and Me9H3, and a redistribution of the nuclear envelope protein LBR in MADA cells. These cells showed evident alterations of the nuclear periphery at the interface between peripheral heterochromatin and the nuclear envelope. Interestingly, the degree of morphological alterations correlated with patient’s age. In fact, fibroblasts derived from the oldest patient (MADA-3) revealed a more pronounced irregularity in envelope organization and heterochromatin distribution. This finding further supports a key role of lamins in...
chromatin organization and mechanical integrity of the nucleus, crucial to maintaining cell and tissue integrity during aging (29). In this context, a recent study provided a molecular link between cellular senescence and heterochromatin structure (33). These authors showed that senescent human fibroblasts accumulate a distinct chromatin structure enriched with heterochromatin proteins, designated SAHF, that excludes active transcription and is characterized by the accumulation of Me9H3 and HP1 proteins. Interestingly, we observed Hoechst-transcription and is characterized by the accumulation of a distinct chromatin structure enriched with heterochromatin proteins, designated SAHF, that excludes active transcription and is characterized by the accumulation of Me9H3 and HP1 proteins. Interestingly, we observed Hoechst-positive foci highly resembling SAHF in ~15% of MADA-1 nuclei with HP1β and methylated histone H3 at lysine 9 concentrated in these foci, suggesting a process of accelerated cellular senescence in these cells. In agreement with this observation, we found an increase in senescence-associated β-galactosidase staining in MADA cells, which correlates with patient’s age (data not shown). Moreover, we demonstrated that HP1β and Me9H3 become partially solubilized by Triton X-100 treatment, consistent with the finding that heterochromatin in these cells is partly unstructured. This was also confirmed by the fact that Me9H3 loses its intracellular localization in 30% of MADA-3 nuclei. In accordance with the histone code hypothesis, Me9H3 is required to create high-affinity binding sites for HP1, crucial to promote the formation of higher-ordered heterochromatin structures (4, 13, 47). This observation may help explain the dramatic loss of heterochromatin areas and the nuclear lamina thickening in MADA nuclei. In fact, the ultrastructural microscopy shows progressive alterations in nuclear architecture in fibroblasts obtained from MADA patients bearing the common R527H mutation.

Focal loss and, in many cases, detachment of peripheral heterochromatin and alteration of nuclear morphology (nuclear envelope invaginations and/or papillary ektrofissures), similar to those found in other laminopathies, were observed (2, 8, 15, 19, 26, 32, 34, 40, 41, 44, 45). These changes, together with the altered distribution of the two major heterochromatin components, Me9H3 and HP1β proteins, strongly support the hypothesis that R527H mutation may alter the normal formation of heterochromatin-nuclear lamina protein complex. Chromatin defects observed in MADA nuclei are comparable with those observed in the nuclei from lamin A/C−/− mouse fibroblasts (44) and with alterations shown in EDMD, FPLD, and HGPS nuclei (9, 19, 37, 41). However, at least two features are exclusively found in MADA and HGPS nuclei: the complete absence of heterochromatin areas and the nuclear lamina thickening (present study and Ref. 19). Both of these nuclear defects could be related to the accumulation of unprocessed lamin A precursor, as observed in HGPS (14, 19) and MADA cells (present study and Ref. 10). Altered prelamin A processing and defective nuclear envelope organization have also been demonstrated in Zmpste24-deficient mice (6, 38). Interestingly, Fong et al. (16) recently demonstrated that the accumulation of prelamin A is responsible for many aspects of the disease-associated phenotype, including the misshapen nuclei, and that lowering the prelamin A level may modify the evolution of the disease (16). In this context, notwithstanding the fact that heterozygous R527H cells exhibit nuclear abnormalities (35), we did not observe any significant increase of prelamin A level with respect to age-matched controls (data not shown).

We observed a marked redistribution of LBR in MADA cells. Mislocalization of LBR was also reported in EDMD2 fibroblasts and in myoblasts, suggesting that lamin A/C mutations, directly or indirectly, affect the localization of the nuclear envelope protein LBR (40). Moreover, several studies have highlighted the importance of the association of LBR with components of the heterochromatin such as HP1 proteins (25, 39, 48). Therefore, our study provides a link between lamin A mutations and altered chromatin remodeling, supporting a common pathogenetic mechanism. Recent findings suggest that the multisystem nature and the wide spectrum of phenotype variation of several monogenic disorders are attributable to defects of chromatin remodeling (3, 7, 12, 21). Laminopathies represent an excellent model to investigate the molecular basis of this phenomena. In fact, it is not clear why mutations in LMNA, EMD, and LBR, which are expressed in most cells, cause tissue-specific disorders. On the other hand, it is unclear why different mutations in LMNA cause different diseases (27, 31, 36). Several hypotheses were suggested to explain their pathogenetic mechanisms. These include the mechanical/structural model and the gene expression model (20). Although these models are not mutually exclusive, they do not explain the etiological link between an altered nuclear envelope and transcriptional misregulation. Dramatic defects in nuclear envelope structure are evident in cells from patients with EDMD, FPLD, or progerias and in mice carrying engineered mutations in LMNA. In particular, the nuclei show frequent blebbing or “herniations” with evident alterations in nuclear shape, increased separation of the inner and outer nuclear membranes, clustering of nuclear pores, loss of some inner nuclear membrane proteins from one pole of the nucleus, and disruption of the underlying electron-dense heterochromatin (9, 19, 30, 37, 41, 44). Nuclear envelopes from Lmna−/− mice exhibit increased fragility (2, 26, 34), and, in general, nuclei containing defective lamins may be mechanically more fragile. Our study provides the first evidence of an alteration of heterochromatin-associated protein distribution in laminopathies and allows the first direct correlation between worsening of lamin A defect (precursor protein accumulation) and increasing heterochromatin loss. Provided that HP1β, LBR, and Me9H3 belong to the same functional complex and all appear affected in MADA cells, our results argue for a role of lamin A in the correct assembly and/or stability of this chromatin-associated complex. Moreover, mislocalization of emerin was also observed in MADA-3 cells, the cells obtained from the oldest patient showing major nuclear defects. It is noteworthy that absence of interaction between emerin and lamin A was previously found in FPLD fibroblasts (9), which also accumulate prelamin A (10).

The phenotypic variations associated with mutations in the LMNA gene reflect the functional diversity, redundancy, and modulation of the lamin maturation process in different cellular types. As a consequence, the spectrum of mutations that affect lamin-protein interaction could give rise to multiple phenotypes, because each mutation could differentially affect this pathway. Additionally, slight variations in the function of redundant and cooperative pathways could also contribute to the phenotypic diversity. For example, because the regulation of gene expression requires a fine compartmentalization that is supported by chromatin architecture, mutations in different lamin sites could generate an alteration in gene transcription. Our results provide further support for the hypothesis of a regulatory pathway connecting, in sequence, cellular mor-
phometry, nuclear architecture, chromatin structure, and gene expression.

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